STUDIES ON SYNTHESIS AND APPLICATION OF SUPERPARAMAGNETIC NANOPARTICLES IN RADIOTHERAPY

A Synopsis of the Ph.D. Thesis
Submitted to the Mangalore University

For the award of the degree of
DOCTOR OF PHILOSOPHY IN PHYSICS

Submitted by
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SYNOPSIS OF THE DOCTORAL THESIS

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Studies on Synthesis and Application of Superparamagnetic Nanoparticles in Radiotherapy

Introduction

Superparamagnetic nanoparticles (SPMNPs) are being used as the agents of drug and gene delivery, MRI imaging, and hyperthermia in cancer applications [1]. They are also capable of enhancing radiation damage when placed in cancer cells during radiotherapy. This technique is known as nanoparticle radiosensitization [2]. The damage of normal cells is one of the major limitations of radiotherapy. The nanoparticle radiosensitization helps in overcoming this limitation by minimizing the damage to normal cells.

In general, the SPMNPs are made up of moderate-Z (Z-atomic number) elements such as iron, manganese, cobalt, nickel, copper, and zinc. The radiosensitization potential of the SPMNPs is less studied compared to high-Z nanoparticles (NPs) such as gold [3] and gadolinium [4] due to the notion that only high-Z materials can enhance radiation damage through the mechanism of physical effect [5]. The physical effect is an effect that results from the secondary electrons generated by the interaction of ionizing radiation with the atoms of the NPs. The high energetic secondary electrons, while moving through the cytoplasm, produce reactive oxygen species (ROS) and thus induce oxidative stress in the cells. The oxidative stress can lead to cell death by either necrosis or apoptosis. However, recent studies showed that physical effect is not the only mechanism through which the NPs enhance radiation damage in cancer cells [6,7]. There are also chemical and biological mechanisms that contribute to the radiosensitization and they depend on the chemical nature of elements of NPs and not on their atomic number. This suggests that the radiosensitization potential of the NPs should be evaluated irrespective of their atomic number.

Gadolinium is used as a radiosensitizer because it can also support T_1-weighted MRI imaging [8]. Similarly, $MnFe_2O_4$, Fe_3O_4, $CoFe_2O_4$, $NiFe_2O_4$, $CuFe_2O_4$, and $ZnFe_2O_4$ ferrites are known to support T_2 weighted MRI imaging [9]. Therefore, the radiosensitization study of these moderate-Z NPs becomes important in the context that if they proved to be good radiosensitizers then a single NP can fulfill the requirements of cancer theragnostic. Thereby, the side effects, which could have been caused by the usage of multiple NPs, can be reduced.

Moreover, there is a possibility that the SPMNPs utilized for other cancer applications may be present in the cancer cells during radiotherapy, which would produce radiosensitization and in turn influence the results of radiotherapy. Therefore, the present study also assesses the risk associated with these NPs, if any, when used prior to radiotherapy. So far, there are only limited studies available on the radiosensitization of SPMNPs. A detailed study is necessary beforehand for any NPs to be taken to preclinical or clinical study.

In the present investigation, $Zn_{0.4}M_{0.6}Fe_2O_4$ (where M = Mn, Co, Ni, Cu, and Zn) SPMNPs were studied. The different divalent metal ions were chosen as M for two reasons. Firstly, to understand how different ions (M) influence the radiosensitization potential of the SPMNPs. Secondly, to study the variation of saturation magnetization (M_s) of the SPMNPs as a function of M. M_s is an important parameter of SPMNPs that can affect the efficiency of MRI imaging and hyperthermia. As we are employing bare NPs, the study would also shed light on the risks connected with using these NPs in cancer therapy. The rationale for employing bare NPs is that enzymes present in the cellular environment will eat up any capping agent used on the NPs, exposing the NPs surface to the cellular environment. Moreover, the chemical and biological effects of elemental composition cannot be verified when a capping agent is used, as the observed enhancement will be attributable to the capping agents.

A possible candidate for radiosensitization application in low-Z NPs is reduced graphene oxide (rGO). Because, in the case of metal or metal oxide NPs, suitable capping agents are required to make them biocompatible and drug delivery agents. Whereas, rGO has intrinsic oxygen functional groups. However, rGO synthesized using conventional techniques has shown toxicity because of the residual toxic precursors used in its preparation. Therefore, the present study aims to prepare rGO using a nontoxic chemical precursor (Glucose) and to evaluate its toxicity and radio-modification ability. We employ the bottom-up approach for the rGO synthesis [10]. Moreover, there are no studies regarding the toxicity and radio modification ability of bottom-up synthesized rGO. This is the first study of its kind. If the glucose-derived rGO (g-rGO) possesses all the useful features of the conventional rGO and proved nontoxic then it can make cancer therapy cost-effective. This would also motivate researchers to test the radiosensitization potential of other graphene oxide-related materials synthesized via the bottom-up approach.

The rGO/SPMNPs nanocomposite is useful in biomedical applications [11]. Their radiosensitization application studies are rare. Individually, both have their unique way of

enhancing radiosensitization. However, the way they would complement each other when used in combination is not known. Therefore, in the present investigation, the synergetic radiosensitization effect of the rGO/SPMNPs nanocomposite was studied. The rGO derived using glucose was used for the preparation of the rGO/SPMNPs nanocomposite. Overall, the study aims to evaluate the radiosensitization ability of the moderate-Z and low-Z NPs. This *in vitro* study would be the first step toward clinical testing of these nanomaterials.

Aim and Objectives

The present investigation aims to synthesize, characterize and evaluate cytotoxicity, anticancer and radiosensitization potential of superparamagnetic nanoparticles (SPMNPs), glucose-derived reduced graphene oxide (g-rGO), and g-rGO/SPMNPs nanocomposite.

The objectives are as follows:

- Synthesis and characterization of $Zn_{0.4}M_{0.6}Fe_2O_4$ (where M = Mn, Co, Ni, Cu, and Zn) SPMNPs by hydrothermal method.
- Evaluation of cytotoxicity and anticancer potential of $Zn_{0.4}M_{0.6}Fe_2O_4$ SPMNPs against astrocytes and HT-29 colon cancer cells respectively by MTT assay.
- Assessment of radiosensitization potential of $Zn_{0.4}M_{0.6}Fe_2O_4$ SPMNPs by clonogenic cell survival assay.
- Synthesis and characterization g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite by hydrothermal method.
- Evaluation of cytotoxicity and anticancer potential of g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite against astrocytes and HT-29 colon cancer cells respectively by MTT assay.
- Assessment of radiosensitization potential of g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite by clonogenic cell survival assay.

The Thesis

The thesis has been divided into four chapters. Chapter 1 introduce NPs and the rationale behind nanoparticle radiosensitization, Chapter 2 describes the various materials and methods used for the preparation, characterization, cytotoxicity, anticancer, and radiosensitization study of the NPs, Chapter 3 presents results and discussion, and Chapter 4 summarizes all the major results and also provides conclusions and suggestions for future studies.

Chapter 1: Introduction

The chapter starts with the general introduction to the present investigation and briefly introduces NPs used in the study i.e. $Zn_{0.4}M_{0.6}Fe_2O_4$ (where M = Mn, Co, Ni, Cu, and Zn) SPMNPs and g-rGO, colon cancer and its treatment methods, radiotherapy and its limitations, radiosensitization, and rationale behind nanoparticle radiosensitization. A detailed literature review on the radiosensitization application of the NPs and various parameters affecting the same is presented in the chapter. It also contains the importance of the present research work. At the end of the chapter, the aim and objectives of the current investigation are presented along with the thesis structure.

Chapter 2: Materials and Methods

This chapter describes the materials and methods used in the preparation, characterization, and radiobiological studies of the NPs. All the NPs studied in the present investigation were prepared using the hydrothermal method. Figures 1, 2, and 3 present the schematic diagram of the preparation of $Zn_{0.4}M_{0.6}Fe_2O_4$ (where M = Mn, Co, Ni, Cu, and Zn) SPMNPs, g-rGO, and rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite, respectively.

The NPs were characterized using the following techniques. The crystalline structure, size, lattice constants, and cationic distribution of the prepared sample were investigated by X-ray diffractometer (XRD, Rigaku Miniflex600) with Cu K-α radiation (λ=1.54 A°) at a scan rate of 2°/min, in the scan range of 10°- 80°. The elemental composition and cationic distribution of the sample were identified using an X-ray photoelectron spectrometer (XPS, KRATOS Axis Ultra DLD) having a monochromatic Al K-α X-ray source (1486.6 eV).

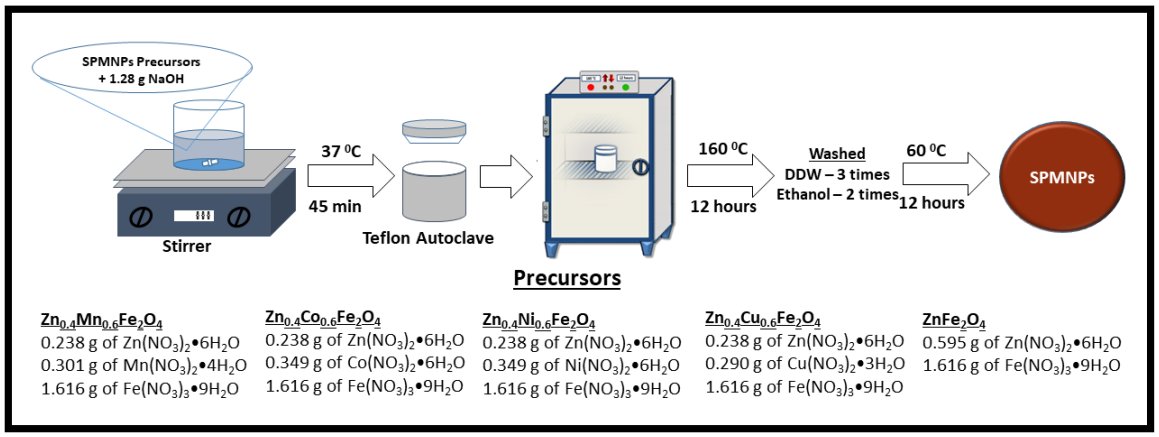

Figure 1. Schematic diagram of the synthesis of SPMNPs by Hydrothermal Method.

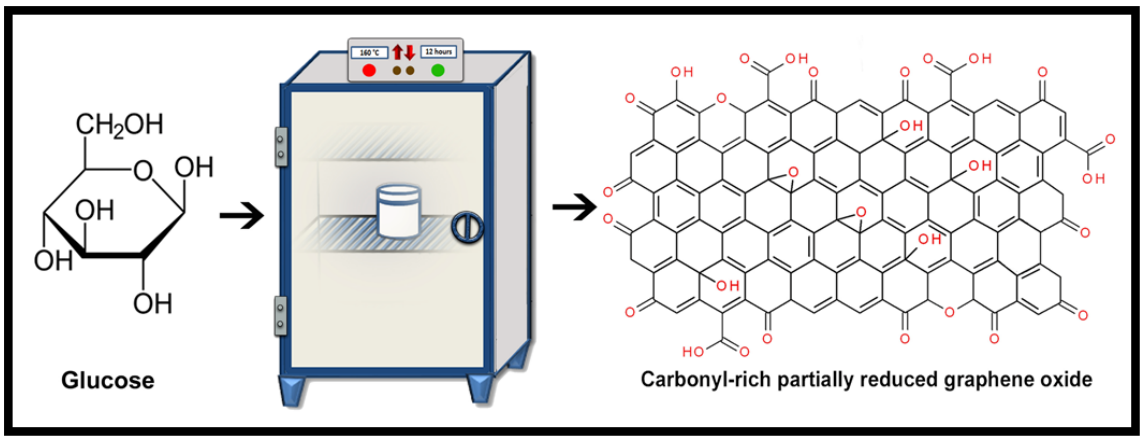

Figure 2. Schematic diagram of the synthesis of glucose-derived rGO by Hydrothermal Method.

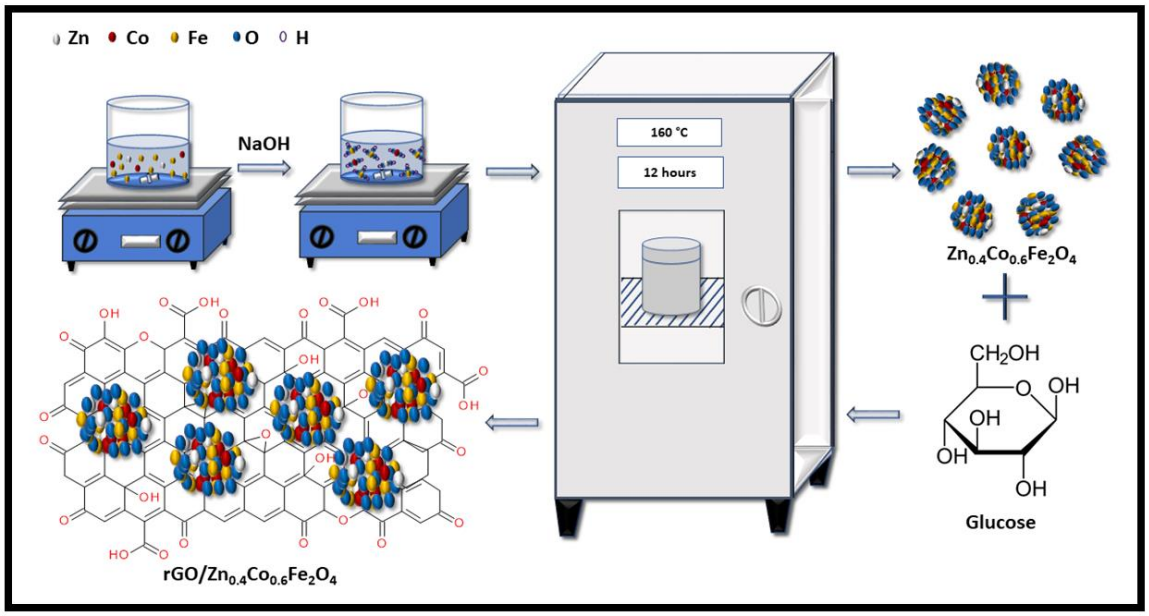

Figure 3. Schematic diagram of the synthesis of rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ SPMNPs by Hydrothermal Method.

The magnetic property of the sample was studied using a vibrating sample magnetometer (VSM, Quantum Design) with an applied magnetic field of - 40 kOe to + 40 kOe, at room temperature. The surface functional groups were identified using a Fourier-transform infrared spectrometer (FTIR, SHIMADZU IR Prestige-21) in a wavenumber range 4000-500 cm^{-1}. UV-Vis absorption spectra were recorded using SHIMADZU 1800 UV-Vis spectrometer in the wavelength range of 190 to 800 nm. Raman spectroscopy measurements were taken using a Raman microscope (Raman, HORIBA LabRAM HR) with a 532 nm laser. The morphology of the sample was observed using a field emission-scanning electron microscope (FE-SEM, Carl Zeiss Sigma). Particle size and zeta potential were measured by the BROOKHAVEN Zeta PALS zeta potential analyzer.

The radiobiological studies of the NPs were carried out using the astrocyte normal cells and HT-29 colon cancer cells. The cytotoxicity of the NPs against astrocytes and anticancer potential of the NPs against HT-29 colon cancer cells was studied by MTT assay. The radiosensitization potential of the NPs against HT-29 colon cancer cells was studied by clonogenic cell survival assay. A Co-60 gamma source was used for gamma irradiation with a radiation dose of 2 Gy. ANOVA was used to study the statistical significance of the results of biological studies.

Chapter 3: Results and Discussion

Chapter 3 is divided into four sections. Section 3.1 and 3.2 deal with characterization and radiobiological studies of SPMNPs, respectively. Section 3.3 and 3.4 deal with the characterization and radiobiological studies of g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite.

3.1: Characterization of the superparamagnetic nanoparticles

The chapter presents the physicochemical properties of the SPMNPs as studied by various characterization techniques. The SPMNPs were characterized using XRD, XPS, VSM, FTIR, FE-SEM, and DLS techniques. The XRD characterization showed the formation of highly crystalline NPs with cubic spinel structure. The average crystallite size of the SPMNPs calculated by the Debye-Scherrer equation using the (311) plane varies from 5.61 to 10.37 nm. The lattice constants vary from 8.4 to 8.53 Å. The chapter provides the cationic distribution results of the SPMNPs as studied by the Rietveld refinement technique. XPS characterization confirmed the stoichiometry of the prepared SPMNPs. The chapter also provides the cationic

distribution results of the SPMNPs as studied by XPS. VSM results confirmed that all the NPs are superparamagnetic and their saturation magnetization value ranges from 21 to 73 emu/g. As observed by FTIR characterization the surface of the NPs contains a small amount of hydroxyl and carbonyl functional groups. FE-SEM characterization showed that the $ZnFe_2O_4$, $Zn_{0.4}Cu_{0.6}Fe_2O_4$, and $Zn_{0.4}Ni_{0.6}Fe_2O_4$ NPs have spherical morphology whereas $Zn_{0.4}Mn_{0.6}Fe_2O_4$ and $Zn_{0.4}Co_{0.6}Fe_2O_4$ NPs shows a combination of rod-like and spherical morphology. The hydrodynamic mean size of the SPMNPs as calculated by DLS characterization ranges from 47.2 to 68.1 nm and zeta potential ranges from 26.8 to 34.7 mV. The chapter also presents a possible synthesis mechanism at the end.

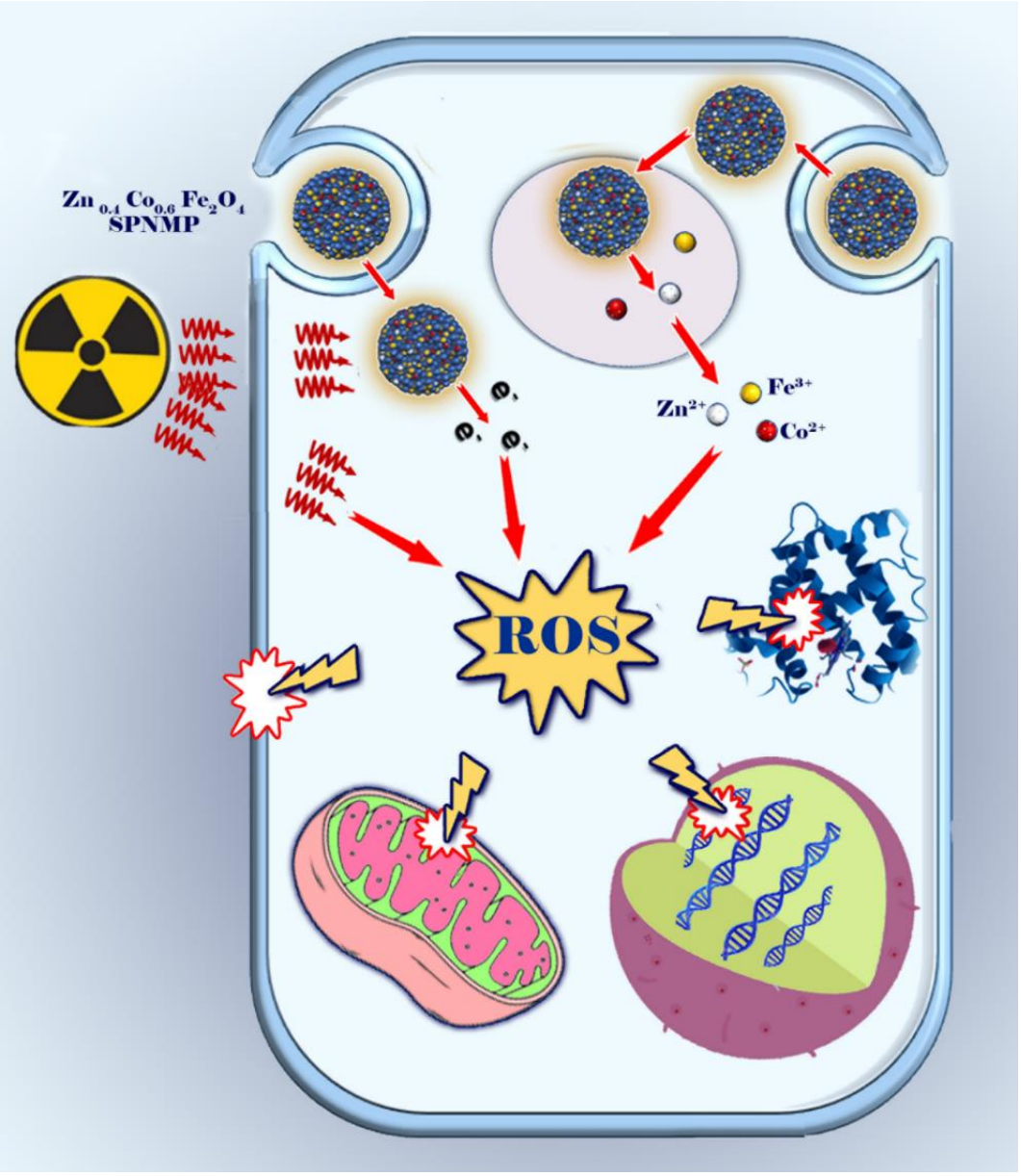

Figure 4. Cytotoxicity, anticancer, and radiosensitization mechanism of SPMNPs.

3.2: Radiobiological studies of superparamagnetic nanoparticles

The chapter deals with the results and discussion of the radiobiological studies of SPMNPs. The SPMNPs exhibited a dose-dependent decrease in cell viability in both normal and cancer cells. In normal cells, the percentage cell viability was reduced to 80% at a high concentration of 200 μg/mL. In cancer cells, the percentage cell viability was reduced to 60% even at a low concentration of 12.5 μg/mL and to 90% at 200 μg/mL. This suggests that SPMNPs are more toxic to cancer cells compared to normal cells. The cancer cells treated with SPMNPs and γ-radiation showed damage enhancement in the range of 1.87 - 3.67 fold compared to cells treated with γ-radiation alone. The chapter also provides a possible mechanism of cytotoxicity, anticancer, and radiosensitization potential. Figure 4 provides a schematic representation of radiosensitization mechanisms of SPMNPs.

3.3: Characterization of g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite

The chapter presents the physicochemical properties of g-rGO and g-rGO/ $Zn_{0.4}Co_{0.6}Fe_2O_4$ as studied by various characterization techniques. The NPs were characterized by XRD, XPS, VSM, FTIR, UV-Vis, Raman, SEM, and DLS techniques. The characterization confirmed the formation of desired NPs. The g-rGO contains 2-3 layers with an interlayer spacing of 3.95 Å. The I_D/I_G ratio of the sample is 1.39. It has a flake-like structure with a hydrodynamic mean size of 654.2 nm having a moderate zeta potential of -27.40 mV. In g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite, g-rGO has an interlayer spacing of 3.4 Å. I_D/I_G ratio of the sample is 0.98. It has a flake-like structure and hydrodynamic size of 616.2 nm with a moderate zeta potential of -26.52 mV. The saturation magnetization of the sample is 29 emu/g. The present work offers a simple, cost-effective, and eco-friendly method for the mass production of g-rGO and g-rGO/SPMNPs nanocomposite. The chapter also presents a plausible reaction mechanism of synthesis of g-rGO (Figure 5).

3.4: Radiobiological studies of g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite

The chapter presents the results and discussion of the radiobiological studies of g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite. The g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite were nontoxic to astrocyte normal cells up to 100 μg/mL. Whereas, they showed around 80% toxicity in cancer cells at a concentration of 100 μg/mL. This suggests that g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ are nontoxic to normal cells and toxic to cancer cells at a concentration of 100 μg/mL. The cancer cells treated with both g-rGO and γ-radiation showed damage

enhancement of 2.55 fold compared to cells treated with γ-radiation alone. The cancer cells treated with both g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ and γ-radiation showed damage enhancement of 3.8 fold compared to cells treated with γ-radiation alone.

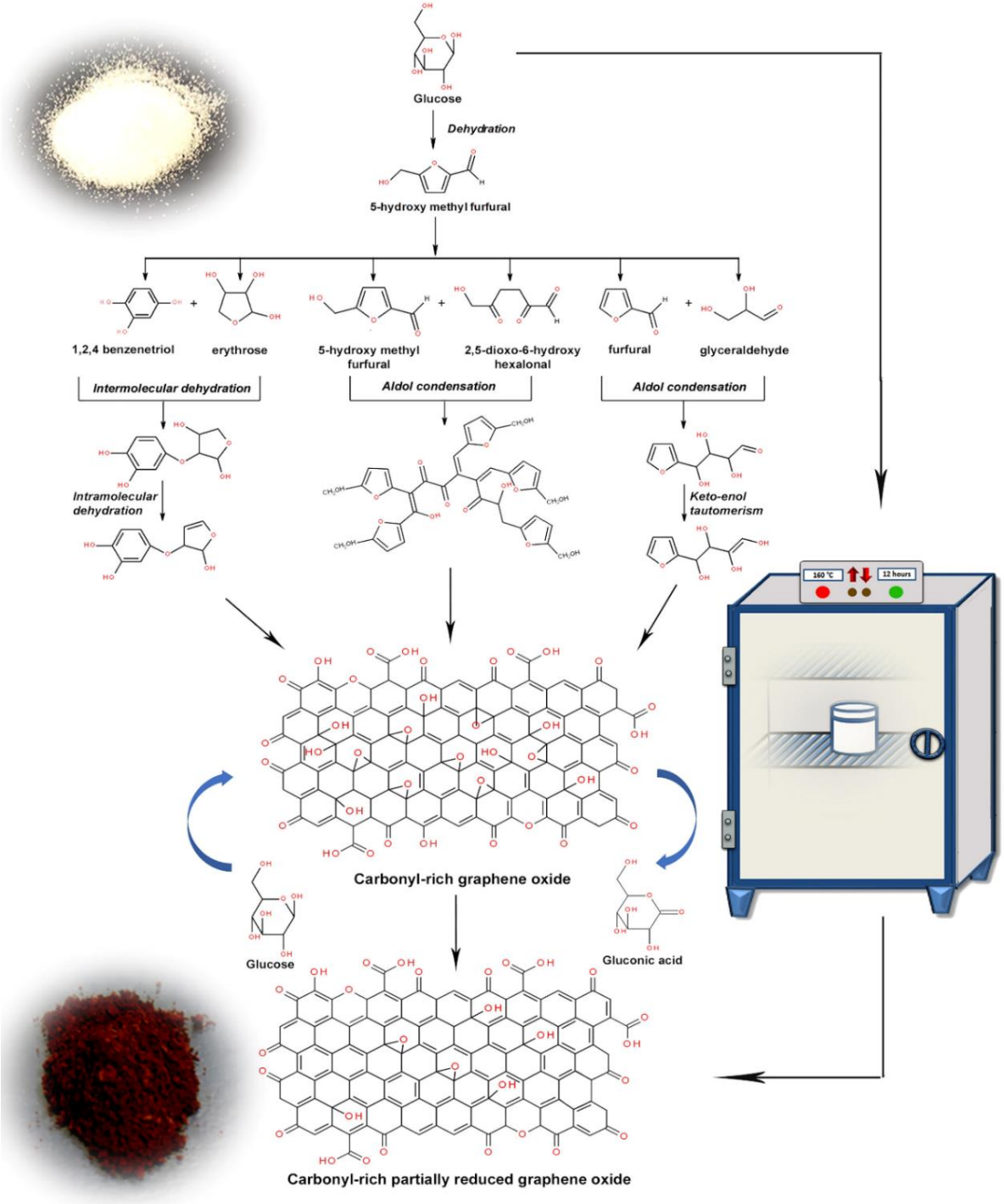

Figure 5. Reaction mechanism showing the synthesis of glucose-derived rGO by hydrothermal method

Chapter 4: Summary and Conclusions

The chapter provides the summary of all the results and discussion presented in chapter 3. The characterization of the SPMNPs using various techniques showed that they have a saturation magnetization of 21-73 emu/g, monodispersity, the zeta potential of 30 mV, and hydrodynamic mean size around 50 nm, which are suitable for biomedical applications. The SPMNPs were more toxic to cancer cells compared to normal cells. The SPMNPs exhibited radiation damage enhancement (radiosensitization) potential with maximum radiation damage enhancement of 3.67 fold for $Zn_{0.4}Mn_{0.6}Fe_2O_4$ NPs. The study also described an unconventional efficient bottom-up method of synthesizing rGO and rGO/SPMNPs nanocomposite using glucose as a carbon source for rGO. The formation of g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite was confirmed using various characterization techniques. Both, g-rGO and g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite were nontoxic to normal cells up to 100 μg/mL and were toxic to cancer cells. g-rGO exhibited radiosensitization potential with a radiation damage enhancement of 2.55 fold whereas the g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite exhibited radiation damage enhancement of 3.8 fold. A synergetic radiosensitization effect was observed in g-rGO/$Zn_{0.4}Co_{0.6}Fe_2O_4$ nanocomposite. In conclusion, the moderate-Z SPMNPs, low-Z g-rGO, and the g-rGO/SPMNPs nanocomposite have the potential to improve the efficacy of radiotherapy and hence the cancer treatment. The chapter also provides suggestions for future studies.

(Y Narayana) (Sunil K C)

Research Guide Research Student

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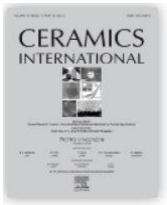
[1] 

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